20-HETE-induced nitric oxide production in pulmonary artery endothelial cells is mediated by NADPH oxidase, H$_2$O$_2$, and PI3-kinase/Akt

Sreedhar Bodiga, Stephanie K. Gruenloh, Ying Gao, Vijay L. Manthati, Narimsahwamy Dubasi, John R. Falck, Meetha Medhora, and Elizabeth R. Jacobs

1Division of Pulmonary and Critical Care Medicine, Medical College of Wisconsin, Milwaukee, Wisconsin; and 2University of Texas Southwestern Medical Center, Dallas, Texas

Submitted 28 August 2009; accepted in final form 4 January 2010

WE HAVE PREVIOUSLY DEMONSTRATED that 20-HETE, a product of arachidonic acid catalyzed by CYP4, induces activation of nitric oxide synthase (NOS) from pulmonary artery endothelial cells in a [Ca$^{2+}$]-dependent manner (9, 23, 36). Furthermore, 20-HETE releases NO, which contributes to endothelium-dependent vasodilation in pulmonary arteries (9, 19). Growing evidence from our own work as well as that from others indicates that 20-HETE activates the vascular NADPH oxidase, leading to increased production of superoxide anion (O$_2^-$) and hydrogen peroxide (2, 23, 32). Superoxide serves as a source of other reactive oxygen species including hydrogen peroxide, which may contribute to vascular disease, and/or, in some cases, may have specific signaling properties (15). In particular, it is known that H$_2$O$_2$ potently induces endothelial nitric oxide synthase (eNOS) activity via a Ca$^{2+}$/calmodulin-dependent protein kinase IIJanus-kinase 2-dependent pathway in bovine aortic endothelial cells (6, 12). Furthermore, it has been shown that exogenous H$_2$O$_2$ in micromolar concentrations activates eNOS from porcine aortic endothelial cells to cause endothelial NO release (7, 28). Superoxide may also react with NO in a diffusion-limited fashion to form peroxynitrite. This species is believed to result in the loss of many of the beneficial effects of NO, including vasodilatation.

The present studies were undertaken to explore the relationship between 20-HETE-stimulated superoxide, hydrogen peroxide, or ONOO$^-$ production and eNOS activation or NO release in bovine pulmonary artery endothelial cells (BPAECs). Because 20-HETE activates NADPH oxidase and PI3-kinase (PI3K), we designed experiments to examine the role of these two signaling pathways in 20-HETE-induced NO production. Specifically, we tested the following hypothesis: stimulated release of NO from BPAECs in response to 20-HETE is mediated by 1) activation of NADPH oxidase, 2) generation of superoxide, which is rapidly dismutated to hydrogen peroxide, 3) hydrogen peroxide-mediated activation of PI3K/Akt, and 4) activated Akt-dependent phosphorylation of eNOS with stimulated NO release. We found that 20-HETE significantly stimulates both superoxide and hydrogen peroxide but does not generate peroxynitrite. We further show that 20-HETE-induced hydrogen peroxide, and not superoxide, plays an essential role in subsequent NO production at least in part by modulating the signaling pathways of PI3K/Akt.

MATERIALS AND METHODS

Materials. Wortmannin (cat. no. 681675), LY-294002 (cat. no. 440202), and Akt inhibitor (cat. no. 124017) were obtained from EMD Chemicals, Gibbstown, NJ. Apocynin (cat. no. 178385) was obtained from Calbiochem, Gibbstown, NJ. Polyethylene-glycolated superoxide dismutase (PEG-SOD; S-9549; 685 U/mg solid, 1 unit inhibits rate of reduction of cytochrome c by 50% in a coupled system with xanthine and xanthine oxidase at pH 7.8 at 25°C in a 3-ml reaction volume), PEG-CAT (C-4963; 17,600 U/mg solid, 1 unit inhibits rate of reduction of cytochrome c by 50% in a coupled system with xanthine and xanthine oxidase at pH 7.8 at 25°C in a 3-ml reaction volume), PEG-CAT (C-4963; 17,600 U/mg solid, 1 unit inhibits rate of reduction of cytochrome c by 50% in a coupled system with xanthine and xanthine oxidase at pH 7.8 at 25°C in a 3-ml reaction volume), PEG-CAT (C-4963; 17,600 U/mg solid, 1 unit inhibits rate of reduction of cytochrome c by 50% in a coupled system with xanthine and xanthine oxidase at pH 7.8 at 25°C in a 3-ml reaction volume), PEG-CAT (C-4963; 17,600 U/mg solid, 1 unit inhibits rate of reduction of cytochrome c by 50% in a coupled system with xanthine and xanthine oxidase at pH 7.8 at 25°C in a 3-ml reaction volume), PEG-CAT (C-4963; 17,600 U/mg solid, 1 unit inhibits rate of reduction of cytochrome c by 50% in a coupled system with xanthine and xanthine oxidase at pH 7.8 at 25°C in a 3-ml reaction volume), PEG-CAT (C-4963; 17,600 U/mg solid, 1 unit inhibits rate of reduction of cytochrome c by 50% in a coupled system with xanthine and xanthine oxidase at pH 7.8 at 25°C in a 3-ml reaction volume), PEG-CAT (C-4963; 17,600 U/mg solid, 1 unit inhibits rate of reduction of cytochrome c by 50% in a coupled system with xanthine and xanthine oxidase at pH 7.8 at 25°C in a 3-ml reaction volume), PEG-CAT (C-4963; 17,600 U/mg solid, 1 unit inhibits rate of reduction of cytochrome c by 50% in a coupled system with xanthine and xanthine oxidase at pH 7.8 at 25°C in a 3-ml reaction volume), PEG-CAT (C-4963; 17,600 U/mg solid, 1 unit inhibits rate of reduction of cytochrome c by 50% in a coupled system with xanthine and xanthine oxidase at pH 7.8 at 25°C in a 3-ml reaction volume), PEG-CAT (C-4963; 17,600 U/mg solid, 1 unit inhibits rate of reduction of cytochrome c by 50% in a coupled system with xanthine and xanthine oxidase at pH 7.8 at 25°C in a 3-ml reaction volume), PEG-CAT (C-4963; 17,600 U/mg solid, 1 unit inhibits rate of reduction of cytochrome c by 50% in a coupled system with xanthine and xanthine oxidase at pH 7.8 at 25°C in a 3-ml reaction volume), PEG-CAT (C-4963; 17,600 U/mg solid, 1 unit inhibits rate of reduction of cytochrome c by 50% in a coupled system with xanthine and xanthine oxidase at pH 7.8 at 25°C in a 3-ml reaction volume), PEG-CAT (C-4963; 17,600 U/mg solid, 1 unit inhibits rate of reduction of cytochrome c by 50% in a coupled system with xanthine and xanthine oxidase at pH 7.8 at 25°C in a 3-ml reaction volume), PEG-CAT (C-4963; 17,600 U/mg solid, 1 unit inhibits rate of reduction of cytochrome c by 50% in a coupled system with xanthine and xanthine oxidase at pH 7.8 at 25°C in a 3-ml reaction volume), PEG-CAT (C-4963; 17,600 U/mg solid, 1 unit inhibits rate of reduction of cytochrome c by 50% in a coupled system with xanthine and xanthine oxidase at pH 7.8 at 25°C in a 3-ml reaction volume), PEG-CAT (C-4963; 17,600 U/mg solid, 1 unit inhibits rate of reduction of cytochrome c by 50% in a coupled system with xanthine and xanthine oxidase at pH 7.8 at 25°C in a 3-ml reaction volume), PEG-CAT (C-4963; 17,600 U/mg solid, 1 unit inhibits rate of reduction of cytochrome c by 50% in a coupled system with xanthine and xanthine oxidase at pH 7.8 at 25°C in a 3-ml reaction volume), PEG-CAT (C-4963; 17,600 U/mg solid, 1 unit inhibits rate of reduction of cytochrome c by 50% in a coupled system with xanthine and xanthine oxidase at pH 7.8 at 25°C in a 3-ml reaction volume), PEG-CAT (C-4963; 17,600 U/mg solid, 1 unit inhibits rate of reduction of cytochrome c by 50% in a coupled system with xanthine and xanthine oxidase at pH 7.8 at 25°C in a 3-ml reaction volume), PEG-CAT (C-4963; 17,600 U/mg solid, 1 unit inhibits rate of reduction of cytochrome c by 50% in a coupled system with xanthine and xanthine oxidase at pH 7.8 at 25°C in a 3-ml reaction volume), PEG-CAT (C-4963; 17,600 U/mg solid, 1 unit inhibits rate of reduction of cytochrome c by 50% in a coupled system with xanthine and xanthine oxidase at pH 7.8 at 25°C in a 3-ml reaction volume), PEG-CAT (C-4963; 17,600 U/mg solid, 1 unit inhibits rate of reduction of cytochrome c by 50% in a coupled system with xanthine and xanthine oxidase at pH 7.8 at 25°C in a 3-ml reaction volume), PEG-CAT (C-49
apocynin, 100 U/ml rescein (DCF) (H9261 presence of intracellular hydrogen peroxide, is oxidized in the PBS, and resuspended in 0.5 ml of PBS. Rhodamine 123 fluoresced in serum-free RPMI followed by the addition of a low concentration (1 µM) of DAF-FM diacetate. This low concentration of DAF-FM significantly reduced the background autofluorescence and improved the signal-to-noise ratio of NO detection (22). After loading, cells were rinsed three times with RPMI and then placed on the stage of a Nikon Eclipse TE200 microscope equipped with a TE-FM epifluorescence attachment (Lambda DG-4 from Sutter Instrument) and captured using a Hamamatsu digital camera C4742-95. NO fluorescence was measured using λex = 488 nm, λem = 520 nm. Because NOS generates superoxide instead of NO in the absence of l-arginine (34), excess of l-arginine (3 mM) was added to all solutions used for NO measurements except for the treatments with l-NAME. As with detection of ROS using fluorescent probes, average fluorescent intensity within operator-defined cell borders was recorded using Metamorph version 6.2 (23).

Detection of 3-nitrotyrosine formation in BPAECs. At ~90% confluence, BPAECs were washed with PBS. The cells were then exposed to ethanol (control), 20-HETE (1 µM), or SIN-1 (10 µM) in serum-free RPMI with incubation at 37°C for 30 min. Detection of 3-nitrotyrosine-modified proteins in cell extracts was accomplished using a specific mouse monoclonal anti-3-nitrotyrosine antibody and slot blot immunoassay. Briefly, 3 µg of protein was applied to a Bio-Dot apparatus (Bio-Rad) to nitrocellulose membrane. The membrane was incubated with anti-nitrotyrosine antibody overnight at 4°C, followed by goat anti-mouse IgG horseradish peroxidase conjugate and visualized with enhanced chemiluminescence. In separate experiments, the protein extract (50 µg/lane) was subjected to SDS-PAGE followed by Western blotting to visualize the extent of nitration of proteins in BPAECs treated with ethanol, 20-HETE, and SIN-1.

Western analysis. After stimulation with vehicle or test agents, cells were chilled on ice and washed three times with cold PBS. They were resuspended by scraping in the presence of 0.5 ml of RIPA buffer (cat. no. 20-188; Upstate, Temecula, CA) supplemented with protease inhibitor cocktail. The mixture was kept on ice for 15 min, after which lysates were centrifuged for 10 min at 20,000 g and the supernatants were used for determining protein concentration via the Bio-Rad protein assay kit. Equal amounts of protein (50 µg/lane) were boiled for 5 min in Laemmli sample buffer (161-0737, Bio-Rad) supplemented with 2-mercaptoethanol, resolved on a 10% Tris-HCl SDS polyacrylamide gel (Bio-Rad), and transferred to nitrocellulose membranes as described previously (18, 19). The blots were developed with appropriate primary and matched secondary antibodies conjugated to horseradish peroxidase and visualized using ECL Plus detection reagent. Blots were scanned with an Alpha Image 220 Analysis System, and the relative densities were determined.

Statistical analysis. 20-HETE-stimulated superoxide, H2O2, or NO production, in the presence of vehicle or pharmacological...
inhibitors, was measured in a minimum of 60 cells/group from three independent culture preparations unless indicated otherwise for each condition. The analysis was performed by an individual blinded to the treatment groups. Fluorescence intensity of test cells imaged in the same time frame and manner as test groups was normalized to the mean intensity for vehicle-treated control groups so that experiments from different days and groups of cells might be compared. For Western blots, a minimum of four to five experiments using cells from three independent culture preparations were performed. The ratio of phosphorylated to total protein in the control group was set as 100%, and other groups were normalized to control. Comparisons among groups for all experiments were performed using one-way analysis of variance. When differences were indicated, a Holm-Sidak post hoc test was em-
ployed. Statistical significance was assumed for \( P < 0.05 \). All grouped data shown in the figures are presented as means ± SD.

**RESULTS**

20-HETE stimulates intracellular hydrogen peroxide formation and not peroxynitrite in pulmonary artery endothelial cells. Our previous studies have shown that 20-HETE stimulates superoxide, hydrogen peroxide, and nitric oxide production in BPAECs (9, 23). To further characterize the nature of ROS generated on 20-HETE stimulation, we used DCF fluorescence as an indicator of ROS, in conjunction with PEG-SOD and PEG-CAT. Figure 1A shows changes in intracellular DCF fluorescence as a measure of ROS production 10 min after exposure of BPAECs to 20-HETE (1 \( \mu \)M). There was a significant increase in DCF fluorescence upon 20-HETE stimulation compared with vehicle (ethanol) treatment. Pretreatment with PEG-SOD further increased the DCF fluorescence with 20-HETE. In contrast, PEG-CAT lowered the DCF fluorescence to basal levels, indicating that \( \text{H}_2\text{O}_2 \) is the major ROS detected with DCF.

We also probed cells with DHR123 as an alternate indicator of ROS levels. During the intracellular release of ONOO\(^-\) or \( \text{H}_2\text{O}_2 \), reduced DHR is irreversibly oxidized and converted to the red fluorescent compound rhodamine 123 (25). We first validated changes in dihydrodiamine 123 (DHR) signal in response to \( \text{H}_2\text{O}_2 \) and ONOO\(^-\) treatments in our endothelial cells. An increase in oxidation of DHR in BPAECs was observed in cells treated with either authentic ONOO\(^-\) (100 \( \mu \)M) or ONOO\(^-\) derived from SIN-1 (10 \( \mu \)M), but not with pH-inactivated ONOO\(^-\). Hydrogen peroxide (100 \( \mu \)M) also increased the DHR fluorescence (Fig. 1B). Next we measured the DHR fluorescence stimulated by 20-HETE in the presence of vehicle, PEG-SOD, or PEG-CAT to distinguish \( \text{H}_2\text{O}_2 \) from peroxynitrite. Similar to DCF, PEG-SOD heightened the DHR123 fluorescence in response to 20-HETE, and PEG-CAT attenuated the fluorescence (Fig. 1C). Finally, we tested the effect of gp91phox peptide inhibitor on enhanced DCF fluorescence evoked by 20-HETE (Fig. 1D). The NADPH oxidase blocking peptide, but not the scrambled version, blocked 20-HETE-evoked increased DCF fluorescence, consistent with a role of NADPH oxidase in enhanced ROS production.

Tyrosine nitration of proteins is believed to represent a molecular footprint of peroxynitrite formation. To further explore the nature of ROS produced in BPAECs by 20-HETE, we evaluated the effect of 20-HETE on the formation of 3-nitrotyrosine. Slot blot analysis using an antibody specific for 3-nitrotyrosine revealed no significant differences in intensity between vehicle and 20-HETE-treated endothelial cell protein extracts (Fig. 1E). However, SIN-1 application resulted in an appreciable increase in intensity. These results provide further support for the conclusion that 20-HETE stimulation of BPAECs results in the formation of hydrogen peroxide without increase in the formation of peroxynitrite.

20-HETE-induced Akt activation is dependent on \( \text{H}_2\text{O}_2 \) formation and NADPH oxidase. We have previously shown that 20-HETE increases phosphorylation of Akt at Ser473 (9). To determine the role of superoxide and hydrogen peroxide on Akt activation in response to 20-HETE in BPAECs, phosphorylation of Akt at Ser473 was studied in the presence of PEG-SOD and PEG-CAT during 20-HETE stimulation. As observed in Fig. 2, A and B, 20-HETE increased Akt phosphorylation compared with control. This increase was further enhanced by PEG-SOD and attenuated by PEG-CAT, indicating a role for \( \text{H}_2\text{O}_2 \) in 20-HETE-evoked activation of Akt.

It is known that 20-HETE activates NADPH oxidase and produces superoxide/hydrogen peroxide in BPAECs (23). Therefore, we explored the potential role of NADPH oxidase in Akt activation upon 20-HETE stimulation. Figure 2, C and D, reveals that apocynin, an inhibitor of NADPH oxidase, abrogated the increase in phospho-Akt seen with 20-HETE. Since apocynin is reported to have antioxidant properties in some endothelial cells distinct from effects on NADPH oxidase (16, 29), we also tested the peptide-based inhibitor in this model. Like apocynin, the peptide-based inhibitor, but not the scrambled peptide, blocked 20-HETE-associated increments in Akt phosphorylation (Fig. 2, E and F). Together, these data suggest that hydrogen peroxide derived from NADPH oxidase is an excellent candidate for 20-HETE-stimulated phosphorylation of Akt.

20-HETE-induced phosphorylation of eNOS is dependent on \( \text{H}_2\text{O}_2 \). Since it is known that phosphorylation of Akt often results in phosphorylation of eNOS at Ser1179, we investigated eNOS activation following 20-HETE treatment. 20-HETE significantly increased the phosphorylation of eNOS at Ser1179 within 30 min (Fig. 3, A and B). This increase was not blocked by PEG-SOD. In contrast, PEG-CAT decreased 20-HETE-stimulated eNOS phosphorylation. These data indicate...
Fig. 2. Activation of Akt in response to 20-HETE is mediated by H$_2$O$_2$ and NADPH oxidase. 

A: BPAECs were treated with PEG-SOD (100 U/ml) or PEG-CAT (500 U/ml) for 30 min before 20-HETE stimulation (1 μM, 30 min). Cells were then lysed, and the lysates were analyzed for levels of phospho-Akt (Ser473) and total Akt ($n = 5$ for each group). A representative Western blot of cell lysates probed with phospho-Akt-Ser473 is shown.

B: phospho-Akt and Akt band densities were analyzed by densitometry, and the means ± SE from 5 independent experiments from 2 different isolates of BPAECs were used to calculate the ratio of phospho-Akt/Akt. 20-HETE (gray bar) increases phospho-Akt (Ser473) compared with vehicle control, and this increase is further enhanced by pretreatment with PEG-SOD (gray hatched bar). Removal of H$_2$O$_2$ using PEG-CAT (gray cross-hatched bar) lowers the phospho-Akt levels without altering total Akt levels. *$P < 0.05$ relative to vehicle control (ethanol); **$P < 0.05$ relative to 20-HETE.

C: BPAECs were incubated in serum-free medium containing an inhibitor of NADPH oxidase, apocynin (1 μM), for 30 min before 20-HETE stimulation (1 μM, 30 min). A representative Western blot of cell lysates probed with phospho-Akt-Ser473 is shown ($n = 5$ for each group). D: 20-HETE (gray bar) increases phospho-Akt (Ser473) compared with vehicle control, and this increase is blunted by pretreatment with apocynin (gray hatched bar), likely due to a block in NADPH-derived H$_2$O$_2$. *$P < 0.05$ relative to vehicle control (ethanol; $n = 5$).

E: BPAECs were incubated in serum-free medium with the gp91 peptide inhibitor or the scrambled peptide. A representative Western blot is shown. Like apocynin, the gp91 peptide inhibited the increase in phospho-Akt associated with 20-HETE, whereas the scrambled peptide did not.

F: 20-HETE (gray bar) increases phospho-Akt compared with control, and this increase is blocked by pretreatment with the gp91phox inhibitory peptide. **$P < 0.05$ relative to vehicle control (ethanol $n = 4$).
that H2O2 and not superoxide control 20-HETE-stimulated eNOS activation.

**Phospho-eNOS activation is dependent on PI3K/Akt.** Next we studied the role of PI3K/Akt in activation of eNOS in response to 20-HETE. Treatment of BPAECs with inhibitors of PI3K, wortmannin (WT; 200 nM), or LY-294002 (20 μM) before vehicle or 20-HETE (1 μM, 30 min) stimulation, reduced phospho-eNOS levels without altering total eNOS levels. **C:** BPAECs were incubated for 30 min with vehicle or inhibitors of PI3K, wortmannin (WT; 200 nM), or LY-294002 (20 μM) before vehicle or 20-HETE (1 μM, 30 min) stimulation. A representative Western blot of cell lysates probed with phospho-eNOS-Ser1179 is shown (top) (n = 5 each). Facilitated elimination of H2O2 using PEG-CAT (gray cross-hatched bar) reduced the phospho-eNOS levels without altering total eNOS levels. *P < 0.005 relative to vehicle control (ethanol).

**H2O2 has bidirectional effect on NO production and is sensitive to PI3K inhibitors.** To mimic the increase in intracellular H2O2 upon stimulation with 20-HETE in BPAECs, we added single boluses of H2O2 ranging from 0 to 1,000 μM to the external media, and then tracked NO production using DAF fluorescence. Hydrogen peroxide effected a dose-dependent increase in DAF fluorescence in BPAECs treated with 100–400 μM H2O2 for 30 min. However, with higher concentrations of H2O2, there was a decrease in DAF fluorescence (Fig. 4F). Thus, H2O2 shows concentration-de-
pendent effects on NO generation in BPAECs in a manner that peaked at 400 μM final concentration in the external media. As anticipated, treatment with L-NAME decreased DAF fluorescence elicited by 400 μM H₂O₂ to levels consistent with background. Interestingly, wortmannin to inhibit PI3K also prevented the increase in DAF fluorescence with 400 μM H₂O₂, suggesting that activation of PI3K/Akt is also required for H₂O₂-associated NO production.

PI3K/Akt is required for NO production in response to 20-HETE. To examine if 20-HETE-induced NO production is mediated by PI3K and Akt, BPAECs were pretreated with wortmannin (200 nM), LY-294002 (20 μM), or Akt inhibitor (10 μM) for 30 min and then loaded with DAF. This treatment was followed by stimulation with vehicle (ethanol) or 20-HETE for another 30 min before assessing changes in intracellular NO formation using DAF fluorescence. 20-HETE failed to increase DAF fluorescence in the presence of these inhibitors, suggesting that PI3K/Akt axis is required for eNOS activation and NO production (Fig. 5).

NADPH oxidase is a candidate source for H₂O₂ mediating 20-HETE-stimulated NO production. To identify a potential subcellular source of H₂O₂ that mediates 20-HETE-stimulated

![Graphs and images showing experimental results](attachment:image.png)
NO production, BPAECs were pretreated with apocynin to inhibit NADPH oxidase. BPAECs failed to produce NO in response to 20-HETE when NADPH oxidase was inhibited. We used a second inhibitor of NADPH oxidase, gp91phox ds tat peptide, to block the association of gp91phox subunit with p47phox. Both these inhibitors prevented the increase in NO in response to 20-HETE (Fig. 6, A and B).

Figure 7 shows a schematic representation of 20-HETE-evoked activation of NO release from BPAECs, which accounts for required activation of NADPH oxidase, H2O2 generation, and activation of PI3K, leading to eNOS activation and stimulated NO release.

**DISCUSSION**

20-HETE, the primary arachidonic acid product of CYP4 isoforms, has a number of potent biological effects in a host of cell types (27). Our study adds to the literature regarding 20-HETE signaling in BPAECs with the following new observations: 1) 20-HETE-induced activation of Akt depends on functional NADPH oxidase and H2O2 as opposed to superoxide, 2) 20-HETE-associated phosphorylation of eNOS at Ser1179 and of Akt at Ser473 is driven by H2O2 as opposed to superoxide, 3) 20-HETE-stimulated NO release from BPAECs depends on PI3K/Akt activation, functional NADPH oxidase, and H2O2 as opposed to superoxide, and 4) 20-HETE promotes the formation of H2O2 as well as superoxide, but not ONOO-, in BPAECs. Our previous reports demonstrated 20-HETE induced increases in superoxide production, activation of NADPH oxidase, and enhanced NO release from BPAECs (9, 23). We have also shown that 20-HETE protects against starvation-induced apoptosis in BPAECs and ischemia reoxygenation injury in ex vivo pulmonary arteries in a manner that depends on NADPH oxidase and PI3K and Akt activation (10).

Together then, these data suggest that 1) 20-HETE-induced activation of NADPH oxidase promotes the formation of superoxide, which is rapidly dismutated to H2O2, 2) activation of PI3K/Akt by 20-HETE and phosphorylation of eNOS requires H2O2, and finally, 3) stimulated NO release in response to 20-HETE or its structural and stable analog, 20–5,14-HEDE, and/or enhanced survival are the end result of this signaling pathway. This hypothesis schematic is depicted in Fig. 7.

20-HETE-evoked activation of NADPH oxidase has precedent. Regulation of NADPH oxidase in renal arteries by CYP4 was first reported by Wang et al. (32). Overexpression of CYP4A in Sprague-Dawley rats increased synthesis of 20-HETE in renal interlobar arteries, increased generation of superoxide, and increased expression of gp91. In BPAECs, 20-HETE stimulates production of superoxide in a manner that can be blocked by inhibitors of NADPH oxidase, and is accompanied by p47phox translocation and Rac1 activation (23). Activation of NADPH oxidase is required for 20-HETE-associated prosurvival effects in BPAECs (10). The present studies provide new information regarding 20-HETE-induced signaling through NADPH oxidase in that we demonstrate phosphorylation of both eNOS and Akt in BPAECs is suppressed by pretreatment with two mechanistically distinct inhibitors of NADPH oxidase, apocynin or gp91phox ds tat (Fig. 2, C and E, and Fig. 4, A and C). Although gp91phox ds tat is designed on a NOX2 motif, it may lack specificity and inhibit other NOX isoforms in BPAECs. Our data position NADPH oxidase as a mediator of 20-HETE-evoked activation of both proteins.
Our data also show for the first time that 20-HETE-stimulated H$_2$O$_2$ and not superoxide activates Akt and eNOS in BPAECs. This conclusion is based on the fact that 20-HETE-induced phosphorylation of both of these proteins (eNOS at Ser1179 and Akt at Ser473) is blocked by PEG-Cat and not PEG-SOD. These observations are consistent with previous reports of H$_2$O$_2$-induced activation of Akt in three cell lines and rat aortic vascular smooth muscle cells (30, 31, 33), as well as our own reports of Akt activation by 20-HETE in BPAECs (23). Similarly, activation of Akt by NADPH oxidase in porcine coronary artery endothelial cells has been observed (8).

Various stimuli, including vascular endothelial growth factor (13) and fluid shear stress (11), activate Akt, resulting in promotion of eNOS activity through increased Ser1179 phosphorylation. Phosphorylation of eNOS is widely recognized as a critical regulatory mechanism controlling eNOS activity in response to physiological stimuli (5, 14, 17, 24). H$_2$O$_2$ is a potent stimulus for NO production and is involved in shear-induced stimulation of the eNOS phosphorylation at Ser1179 and the dephosphorylation of Thr495 in bovine aortic endothelial cells (5). NADPH derived hydrogen peroxide is reported to mediate stimulated NO production in bovine aortic endothelial cells (7). Thus 20-HETE-associated stimulated production of hydrogen peroxide leading to phosphorylation of Akt and eNOS in BPAECs fits well into this background of work.

Exogenous H$_2$O$_2$ is reported to mimic the effect of endogenous receptor-induced production of H$_2$O$_2$ and activation of Akt (21, 31, 33). In this regard, intracellular concentrations of H$_2$O$_2$ after a single bolus of H$_2$O$_2$ to the extracellular media are estimated to be 6- to 10-fold lower in the cytosol and 20-fold lower in peroxisomes (3). To assess the effects of H$_2$O$_2$ on NOS-induced NO production in BPAECs, we exposed cells to a range of concentrations of H$_2$O$_2$ for 30 min and followed changes in DAF fluorescence. We observed a bell-shaped response curve, with significant increases in DAF fluorescence at all concentrations above 50 μM, peaking at 400 μM. Our NO concentration response curve to hydrogen peroxide is very similar to that observed by Cai et al. (7) in bovine aortic endothelial cells, but the x-axis in our experiments is extended to show a decrease in NO release at concentrations above 400 μM. Based on all these data, it can be argued that localized physiological concentrations of H$_2$O$_2$ evoked by 20-HETE may be capable of inducing eNOS activation and NO production.

In addition to phosphorylation of Akt and eNOS, our studies indicate that 20-HETE induced an increase in intracellular NO (based on changes in DAF fluorescence, see Fig. 4E) in a manner that depends on H$_2$O$_2$. These data link 20-HETE-stimulated phosphorylation of eNOS to NO release. The correlation between DAF-FM fluorescence and intracellular NO was confirmed by the data from DETANTONOate (a NO donor)-treated BPAECs, which served as a positive control (data not shown), and that of cells treated with L-NAME, which densely blocked 20-HETE-induced DAF signal, which served as the negative control.

Enhanced DAF fluorescence in cells treated with 20-HETE was blocked by NADPH oxidase or PI3K and Akt inhibitors. The observation that PI3K inhibitors wortmannin and LY-294002 blunt 20-HETE-stimulated increase in NO suggests that stimulated eNOS activation is regulated by PI3K/Akt. Inhibition of PI3K also blocked H$_2$O$_2$-stimulated NO production in the same cells. These observations should not be extrapolated to conclusions of direct activation of PI3K or NADPH by 20-HETE as a single mechanism through which enhanced NO is generated in treated cells. Both PI3K and NADPH oxidase are ubiquitous and powerful signaling pathways, such that we expect multiple effects of activation of these signals in BPAECs or other cells for that matter. Very specifically, it is possible that 20-HETE and/or PI3K have
20-HETE and NO in BPAECs

20-HETE

\[ \rightarrow \]

NADPH oxidase

\[ \rightarrow \]

superoxide

\[ \rightarrow \]

Hydrogen peroxide

\[ \rightarrow \]

PI3-kinase/Akt

\[ \rightarrow \]

eNOS

\[ \rightarrow \]

NO

Fig. 7. Hypothesis schematic. A unifying hypothesis depicting the cascade of signaling events in response to 20-HETE that result in the increased production of NO in BPAECs. We speculate that 20-HETE activates NADPH oxidase, which produces superoxide that is rapidly dismutated to H$_2$O$_2$. H$_2$O$_2$ activates PI3K and Akt, which in turn phosphorylates eNOS. Phosphorylation of eNOS at Ser1179 promotes enhanced NO production.

distinct effects on several steps in this signaling cascade including NADPH oxidase, PI3K activation, eNOS phosphorylation, or others. Despite these caveats, these data provide critical links of 20-HETE-induced NO release from BPAECs (36).

The fact that formation of H$_2$O$_2$ is required for 20-HETE-induced eNOS activation has important potential implications for pulmonary vascular cell biology and function. First, these data suggest that enhanced H$_2$O$_2$ formation may serve as an important mediator of 20-HETE prosurvival effects (10, 23). This speculation is consistent with reports that NO derived from eNOS protects cells from oxidative stress by an antioxidant mechanism (26). Also, our studies provide a potential mechanistic basis for the vasorelaxation effect of 20-HETE in pulmonary circulation despite an associated increase in superoxide formation (9, 19). We speculate that 20-HETE relaxes small pulmonary arteries via H$_2$O$_2$-dependent eNOS activation. Based on the lack of 20-HETE-stimulated ONOO$^-$ within the resolution of our assays, and similar profiles of DCF and DHR fluorescence with PEG-SOD or PEG-Cat in response to 20-HETE, we hypothesize that 20-HETE-stimulated superoxide in BPAECs is rapidly dismutated to hydrogen peroxide. In this case, there may be limited opportunity for superoxide interaction with NO and therefore minimal ONOO$^-$ generation. Additional studies to identify mechanisms through which 20-HETE activates NADPH oxidase and promotes survival (NO dependent or independent) are needed.

ACKNOWLEDGMENTS

We thank Dr. Vani Nilakantan, Div. of Surgery, Medical College of Wisconsin, Milwaukee, for the generous gift of 3-nitrotyrosine antibody used in this work.

GRANTS

Financial support was provided by National Institutes of Health Grants HL-49294 (E. R. Jacobs), HL-68627 (E. R. Jacobs), and GM-31278 (J. R. Falck) and the Robert A. Welch Foundation (J. R. Falck).

DISCLOSURES

No conflicts of interest are declared by the author(s).

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